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Evaluation of Auto-oxidation Kinetics of Bovine Hemoglobin via Gas Exchange Circuit at Pseudo-Physiological Conditions

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Abstract

Ferric hemoglobin ($HbFe^{3+}$, metHb) is the oxidized form of the oxygen storage and transport protein ferrous hemoglobin ($HbFe^{2+}$, Hb). Since methemoglobin (metHb)is an oxidized form of native Hb, it cannot bind and release gaseous ligands such as oxygen, nitric oxide (NO) and carbon monoxide and therefore, minute amounts of metHb in the body is ideal. Native Hb can autoxidize to form metHb due to the presence of intracellular oxygen. The levels of metHb need to be monitored for hemoglobin-based oxygen carriers (HBOCs) at pseudo-physiological conditions so they do not harbor any negative side effects for patients. The goal of this project is to determine the rate of metHb conversion from native Hb types due to auto-oxidation in a closed circuit with oxygen impermeable tubing and a gas contactor membrane at pseudo-physiological conditions. A spectral deconvolution program called Alchromy will be used to determine the molar fractions of various species (metHb, oxyHb, deoxyHb, and hemichrome) in solution. From this type of analysis, a kinetic model for metHb formation can be determined for pseudo-physiological conditions.

Acknowledgements

I would like to express my gratitude towards my research advisor, Dr. Andre Palmer for providing the funding and guidance during the course of this honors thesis project. Additionally, I would like to thank my mentor graduate student in the Palmer Lab, Xiangming Gu, who assisted and mentored me in conducting experiments and analyzing data for this project.

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1 Introduction

Methemoglobin is a form of hemoglobin in the ferric state that cannot deliver oxygen to the tissues and less than 1% of the total hemoglobin consists of methemoglobin in a healthy individual [1]. Additionally, methemoglobin shifts the oxygen dissociation curve of oxyhemoglobin to the left, increasing the binding of oxygen to the remaining hemoglobin but reducing the release of it to tissues [2]. If the body sustains critically high methemoglobin levels, this could lead to inadequate amounts of oxygen in the tissues, inducing hypoxia [1, 3]. Methemoglobinemia occurs when there are high concentrations of methemoglobin in the blood and this could lead to symptoms of nausea and tiredness in the long run [4]. The major cause of the formation of methemoglobin is the spontaneous conversion of minute amounts of oxyhemoglobin into methemoglobin through auto-oxidation which will be investigated in this experiment [5]. The rate of auto-oxidation can be used to understand the pathway of methemoglobin formation and try to develop methods of inhibiting that pathway.

The oxidation of hemoglobin into methemoglobin occurs directly and indirectly, but auto-oxidation is the indirect form where iron and oxygen both participate in the process [6]. The auto-oxidation of normal hemoglobin into methemoglobin occurs approximately at the rate of 3% per day in the body but the methemoglobin is also reduced back to oxyhemoglobin to keep a consistent level of around 1% [7]. The conversion between oxyhemoglobin and methemoglobin and vice versa occurs via an oxidation-reduction reaction [8, 5]. The level of intracellular oxygen tension leads to the formation of oxidizing agents and the conversion of normal hemoglobin into methemoglobin [9]. Oxyhemoglobin is in equilibrium with oxygen and hemoglobin but a strong oxidizing agent can convert oxyhemoglobin into methemoglobin completely [8]. The auto-oxidation of hemoglobin usually occurs at oxygen tensions that lead to the deoxygenation of hemoglobin and in the presence of partially deoxygenated hemoglobin [8, 9]. At an oxygen tension of zero, barely

any methemoglobin forms, but the formation of metHb was facilitated by the increase of oxygen tension [10]. When oxyhemoglobin is oxidized to methemoglobin, it leads to the creation of superoxide radicals as well [7, 8, 11]. The superoxide radical produces hydrogen peroxide spontaneously through dismutation, which leads to increased oxidation of normal hemoglobin [6, 8, 11]. Numerous static auto-oxidation studies have been conducted for several HBOCs, however a dynamic auto-oxidation study mimicking pseudo-physiological temperature has not been done. In order for HBOCs to be used in patients, the stability of the substances cannot just be studied for shelf-life but also for its life inside the body and this project will assist in studying those concepts. Therefore, determining the rate of auto-oxidation of hemoglobin into methemoglobin could assist in the experimentation and creation of polymerized hemoglobin, a hemoglobin-based oxygen carrier (HBOC). For physiological analysis of metHb formation, it requires a arterial blood gas (ABG) analyzer which is not as cost effective and is available in hospital labs but not quite accessible for non-hospital research labs [12]. Eventually, these findings can be applied more widely to organ preservation to keep a constant low level of methemoglobin in organs over a long period of time while they are transported.

Methemoglobin is kept at extremely low concentrations in the blood since it has no real function and can lead to hypoxia in tissues. However, auto-oxidation of native hemoglobin into methemoglobin cannot be controlled without understanding the kinetics of the oxidation reaction. This project allows us to test the stability of native Hb and HBOCs at pseudo-physiological conditions in order for the auto-oxidation kinetics to be controlled when put in patients. It is the preliminary part to understanding the function of methemoglobin before reducing agents can be implemented to help stabilize the HBOCs being tested.

An external circuit can be built to determine the rate of auto-oxidation in pseudo-physiological conditions for the oxygenation and deoxygenation of hemoglobin. We hypothesize that the rate of metHb formation is a first order reaction since it depends of

the concentration of native Hb present. This experiment will create a system similar to the respiratory and circulation systems of the body to conduct better stability studies for HBOCs, especially in regards to methemoglobin formation. It will allow us to more accurately assess how these HBOCs will react at pseudo-physiological conditions and allows us to come up with future solutions for controlling auto-oxidation.

2 Materials and Methods

2.1 Materials and Equipment

During the course of this project, several materials were purchased from it and various equipment in the lab were also used. In terms of the equipment for the research project, the main analysis and data collected tools used were the UV-Vis spectrophotometer to take the absorbance readings and the Neofox dissolved oxygen probe to monitor the pO_2 levels. Additional equipment used in closed-circuit were a heated stir plate mix the bHb solution and warm it to 37 °C and a peristaltic pump from Cole Parmer to circulate the bHb solution throughout the circuit.

In order to prepare the bHb solution, a concentrated sample of bHb, approximately 250 mg/mL, and phosphate buffered saline (PBS) were used. The concentrated bHb was prepared via hemoglobin purification procedures, specifically tangential flow filtration (TFF). Silicone and puriflex tubing from Cole Parmer were used for circulating the bHb solution in the system. The gas contactor member used was the 0.5 in.X1 in. hollow fiber 3-M MiniModule gas/liquid exchange module. A gas flowmeter was used to control and monitor the flow rate of N_2 that goes through the gas contactor membrane.

2.2 Methodology

There were several different types of experiments conducted to examine the effect of ambient air and different nitrogen flowrates on the Hb species composition over a 24 hour time period.

The first set of experiments done were to compare static and dynamic autoxidation of bHb. For the static portion of the experiments, a 1 mg/mL sample of bHb was prepared by diluting a concentrated bHb sample with PBS. The 1 mg/mL sample was transferred into a plastic cuvette and placed into the UV-Vis Spectrophotometer to track its absorbance

over a 24 hour period. The spectrophotometer was programmed to read the absorbance of the sample every hour for the 24 hour span. The static autoxidations experiments were conducted three times to obtain three trials and take the average of them in the future. For the dynamic portion of the experiments, a closed-circuit system using silicone tubing was put through a peristaltic pump to circulate the bovine hemoglobin (bHb) in a small plastic Nalgene bottle using appropriate connections to determine the change in metHb levels at 37 °C. Figure 1 shows how the dynamic autoxidation experiment was set up with its different components.

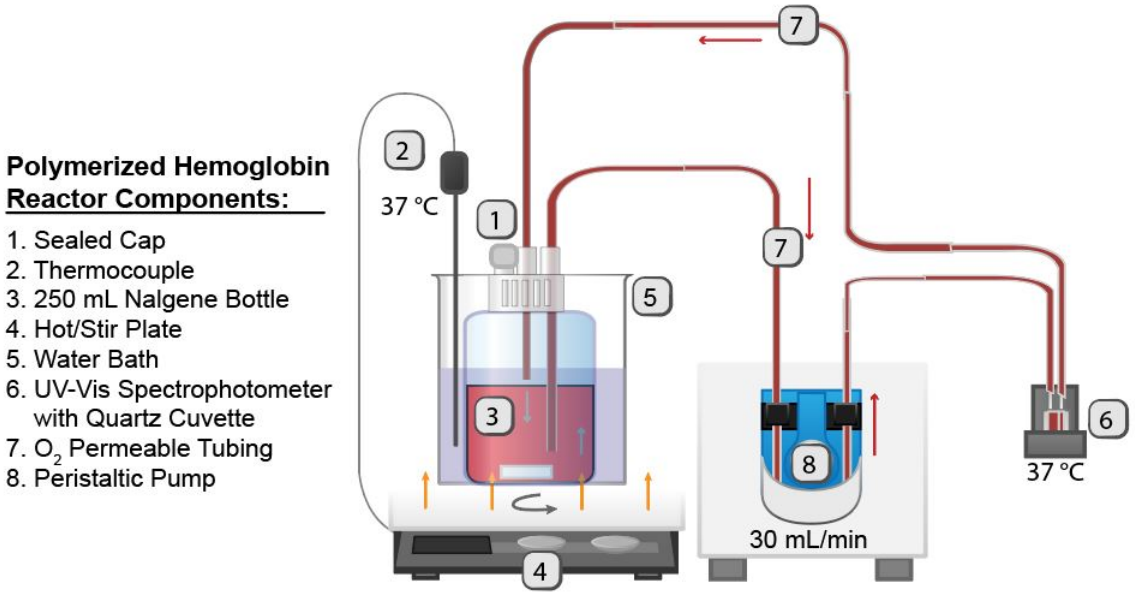

Figure 1: Dynamic autoxidation circuit of bHb solution

The silicone tubing is permeable to oxygen so that the hemoglobin in the tubes can be oxygenated passively by ambient air [13]. The silicone tubing is permeable to oxygen like silicone and it prevents the loss of the sample due to its chemical resistance [14]. The oxygen permeability of silicone platinum-cured tubing that was used is given as 7961 cc-mmsec-cm2-cm Hg x 10^{-10} [14]. The contents of the 250 mL Nalgene bottle was 100 mL of 1 mg/mL bHb sample diluted in a similar manner as the static autoxidation

experiment. The bottle with the bHb solution was placed in a water bath on a heated stir plate to regulate the temperature of the water and the speed of the stir bar circulating. A thermocouple was kept inside the water bath to monitor the temperature so it would be maintained at 37 °C. The tubing connected to the inlet was passed through the pump and attached to the inlet portion of the quartz cuvette. The outlet of the cuvette was attached to the retentate of the Nalgene bottle. The pump was set to 30 mL/min to circulate the bHb solution over the 24 hours. The quartz cuvette portion of the system was placed in the UV-Vis Spectrophotometer and its water bath was also set to 37 °C. The spectrophotometer was programmed to take an absorbance reading every hour for the span of 24 hours. Samples were taken from the bHb solution to test the pO_2 level in the RapidLab machine. This dynamic autoxidation experiment was also conducted a total of three times and the average would be taken for future data analysis. The data from the spectrophotometer for both the static and dynamic autoxidation experiments were then inputted into a spectral deconvolution program, Alchromy, to determine the molar ratios of different species (metHb, oxyHb, deoxyHb, and hemichrome) in the sample. An R script was also developed to analyze the first order reaction rate constant for the change in Hb^{2+} concentrations for both experiment setups.

For the next set of experiments, a closed-circuit system using Puriflex tubing was put through a peristaltic pump to circulate a 1 mg/mL bHb solution in a similar manner as the dynamic autoxidation experiment but a hollow fiber gas exchange membrane was added. The 0.5 in.X1 in. hollow fiber 3-M MiniModule gas/liquid exchange module was used to sweep nitrogen gas (N_2) and actively deoxygenate the bHb solution passing through the circuit. Puriflex tubing was used since it is mostly impermeable to oxygen and the experiment could then focus on just the effect of N_2 and not involve ambient air. The oxygen permeability of the puriflex tubing is given as 200 cc-mmsec-cm2-cm Hg x 10^{-10} [14]. The set up for the experiment is shown below in Figure 2.

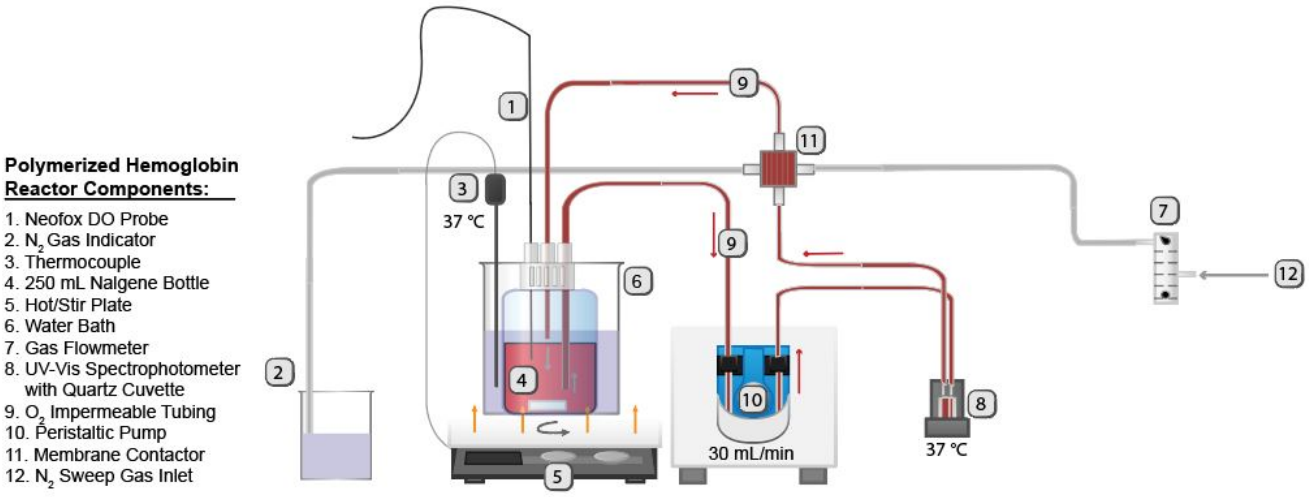

Figure 2: Dynamic autoxidation circuit with N_2 flowing through the gas contactor membrane.

The set up was similar to the dynamic autoxidation experiment but with few connection and tubing changes due to the addition of the gas contactor membrane. The gas contactor membrane was connected to the retentate side of the system to prevent bubbles from passing through the tubing and into the quartz cuvette. If the bubbles were to pass through the quartz cuvette, then this would interfere with the readings taken by the UV-Vis Spectrophotometer. A gas flowmeter was used to monitor and regulate the gas flowrate of N_2. Experiments were conducted at the gas flowrates of 0, 0.25, 0.50, 0.75, and 1.00 mL/min. For each experiment, the spectrophotometer was programmed to take readings every hour for a 24 hour period. The data from the spectrophotometer for these experiments were then inputted into a spectral deconvolution program, Alchromy, to determine the molar ratios of different species (metHb, oxyHb, deoxyHb, etc.) in the sample.

3 Results and Discussion

The data collected from each set of experiments were analyzed through spectral deconvolution methods using Alchromy to determine the fractional compositions of different Hb species. The pO_2 levels were also observed over the 24 hr period to see how they relate to the fractional compositions of different Hb species and N_2 gas flowrates. The reaction rates for particular Hb species such oxyHb were analyzed for the static and dynamic autoxidation experiments.

3.1 Alchromy Spectral Deconvolution Program

Alchromy is a spectral deconvolution program that uses the standard absorbance spectras of different Hb species to calculate the fractional composition of those species in a particular sample. In Figure 3 below, the standard absorbance spectras for each of the Hb species (oxyHb, deoxyHb, metHb, and hemichrome) for wavelengths of 450 to 700 nm are shown.

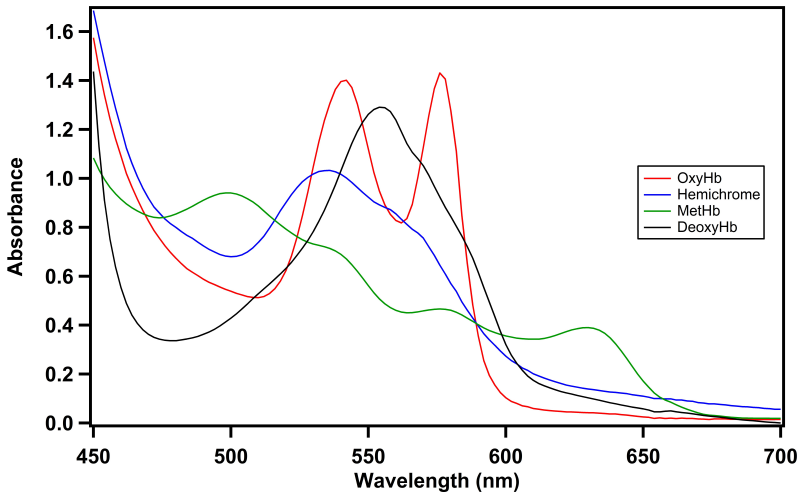

Figure 3: Species absorbance spectras used in Alchromy spectral deconvolution program

The absorbance spectras of the Hb species were compared to the absorbance spectra

found at a particular time point an absorbance reading was taken to determine the fractional composition of each Hb species. The input file for the Alchromy program was an Excel file containing the absorbance readings at each time point. The output of the program is an Excel file with the percent compositions of each Hb species for the different time points, a text file with the initial and final percent compositions, and a graph of the fractional compositions over the time period.

A control experiment was conducted to determine if the Alchromy program was an appropriate analysis tool for estimating the fractional composition of metHb in a solution. A bHb sample almost completely oxidized by nitrite (NO_2) was used to test this concept. The metHb assay used in the research lab is a known assay that is accurate and effective for determining the percent composition of metHb in a sample. A metHb assay was conducted on the sample and Table 2 in Appendix A shows the data obtained from the assay. The output of the Alchromy program gives the initial and final percent compositions of the Hb species and is shown in Figure 9 in Appendix A. It can be seen that the average metHb percent composition for the metHb assay was 94.7% and the final metHb percent composition for the Alchromy program was 97.9955% which are very similar to each other. Since these values are very close to each other, this shows that the Alchromy program is an effective and accurate method to determine the methb percent composition in a sample.

3.2 Static and Dynamic Autoxidation Experiments

The Alchromy program analysis was done for the static and dynamic autoxidation experiments to see how the fractional compositions of the Hb species changed over the 24 hour period. Three trials were conducted for both the static and dynamic autoxidation experiments but the average of those three trials were inputted in the Alchromy program. Figure 4 shows the change in fractional composition of the Hb species (oxyHb, deoxyHb, metHb, and hemichrome) over the 24 hour period for the static and dynamic autoxidation

experiments.

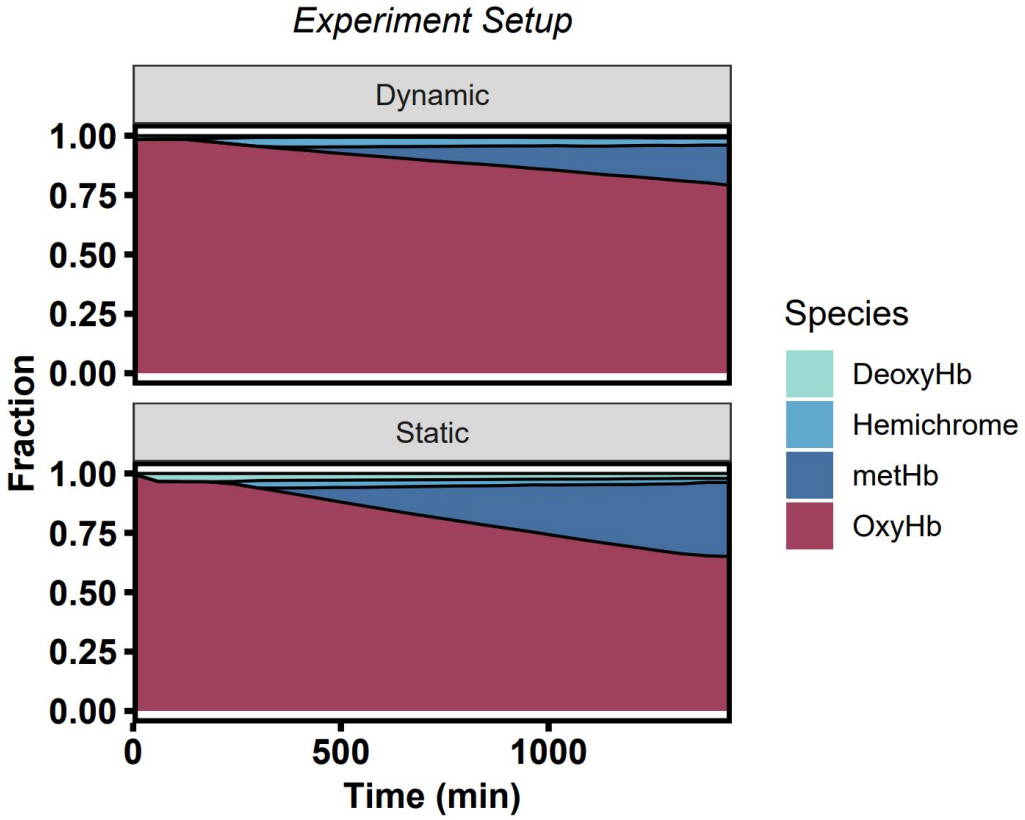

Figure 4: Fractional compositions of Hb species for static and dynamic autoxidation experiments over a 24 hour period.

The fractional compositions of each of the Hb species changed in a fairly similar manner for both the static and dynamic autoxidation experiments. The hemichrome and deoxyHb fractional compositions were very small and consistent compared to the oxyHb and metHb fractional compositions. There was a slightly higher metHb and lower oxyHb fractional compositions for the static autoxidation experiment compared to the dynamic autoxidation experiment.

The rate of bHb autoxidation in a static and dynamic autoxidation experiment were determined by looking at the kinetic rate constants of the $\ln([Hb^{2+}]/[Hb^{2+}]_0)$ over the 24

hour period as shown in Figure 5 below. $[Hb^{2+}]_0$ is given as the initial concentration of the bHb sample that has not been oxidized to the ferric state.

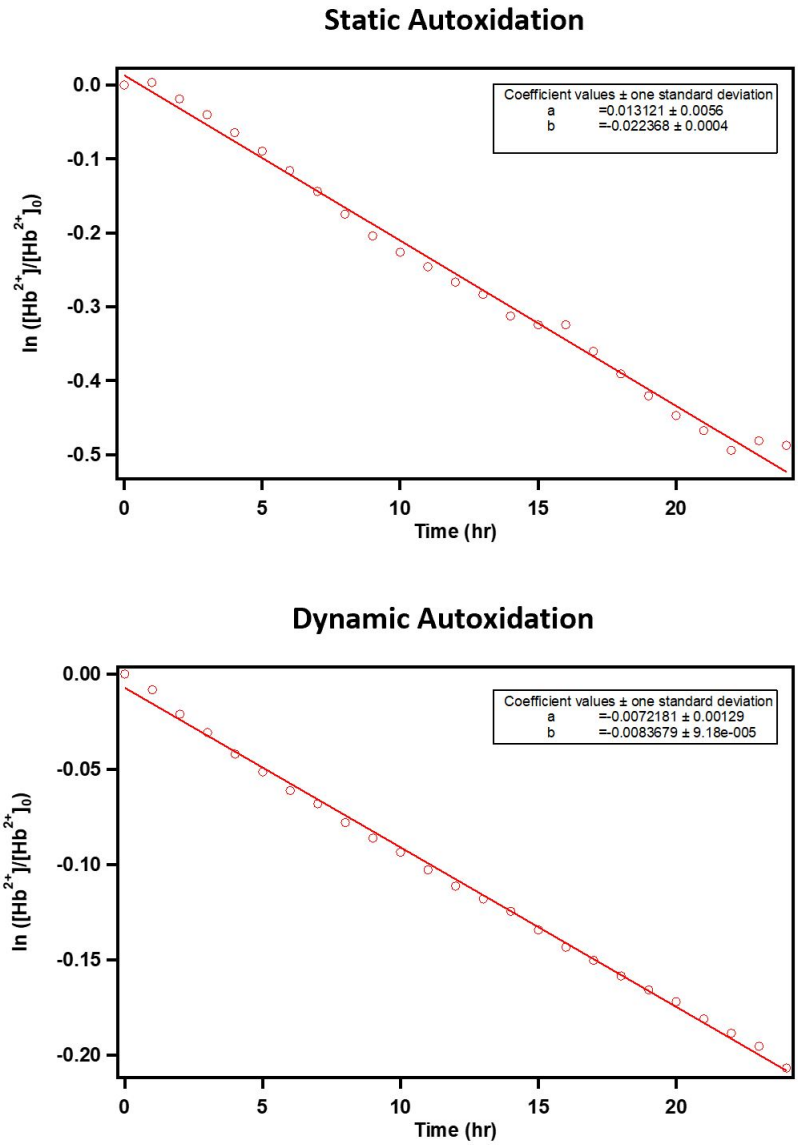

Figure 5: OxyHb change during static and dynamic autoxidation.

Both of the graphs had a very close linear fit, indicating that it is representative of a first order reaction. The kinetic rate constants were a bit higher for the static autoxidation experiment. This corresponds to the higher fractional composition of metHb and lower

fractional composition of oxyHb for the static oxidation experiment. The higher the rate constant the more of the oxyHb is autoxidized and turned into metHb. In order to see if the reaction rate constants between the static and dynamic autoxidation experiments were significantly different, a two-sided t-test was conducted. According to the two-sided t-test assuming unequal variances, the p-value was found to be 0.091 which is greater than the typical significance level of 0.05. Therefore, there is not a significant difference between the reaction rate constants for the static and dynamic autoxidation experiments. These results indicate that both the static and dynamic experiments are good control experiments before adding any deoxygenation procedures.

The pO_2 levels were monitored over the 24 hour period to make sure that the bHb solution was oxygenated using ambient air passing through the silicone tubing. The pO_2 levels for the dynamic autoxidation experiment is shown in Figure 6.

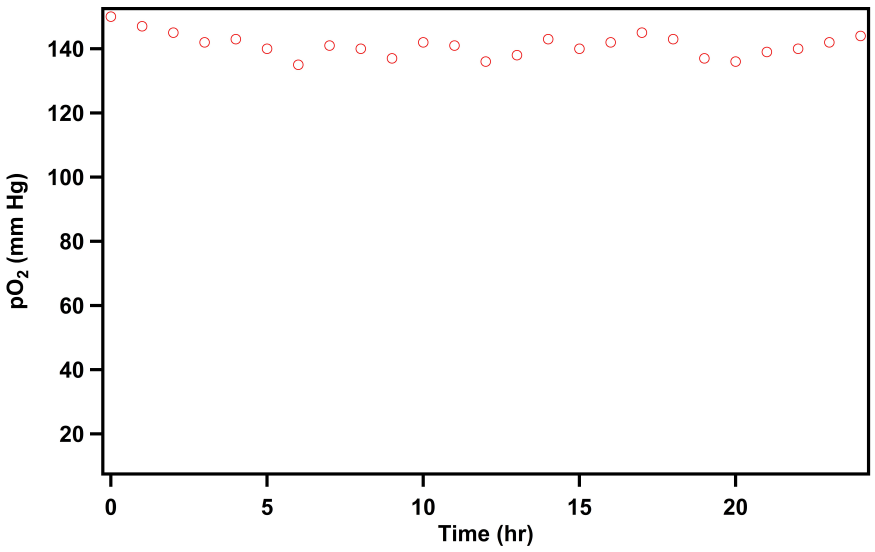

Figure 6: pO_2 levels over the course of the dynamic autoxidation experiment

The pO_2 levels were consistent throughout the course of the experiment. This means that a dynamic equilibrium was reached with the silicone tubing so that the pO_2 levels would stabilize over the 24 hour time frame.

3.3 Autoxidation with Gas Contactor Membrane Experiments

The experiment with the gas contactor membrane sweeping N_2 was used to see how deoxygenation would affect the fraction composition of Hb species (oxyHb, deoxyHb, metHb, and hemichrome) over a 24 hour period. Gas flowrates including 0, 0.25, 0.50, 0.75, and 1.00 mL/min were tested to see how the fractional compositions would differ in each scenario. Figure 7 on the next page shows the fractional compositions of the Hb species for each of the gas flowrates which was developed by the Alchromy program previously mentioned

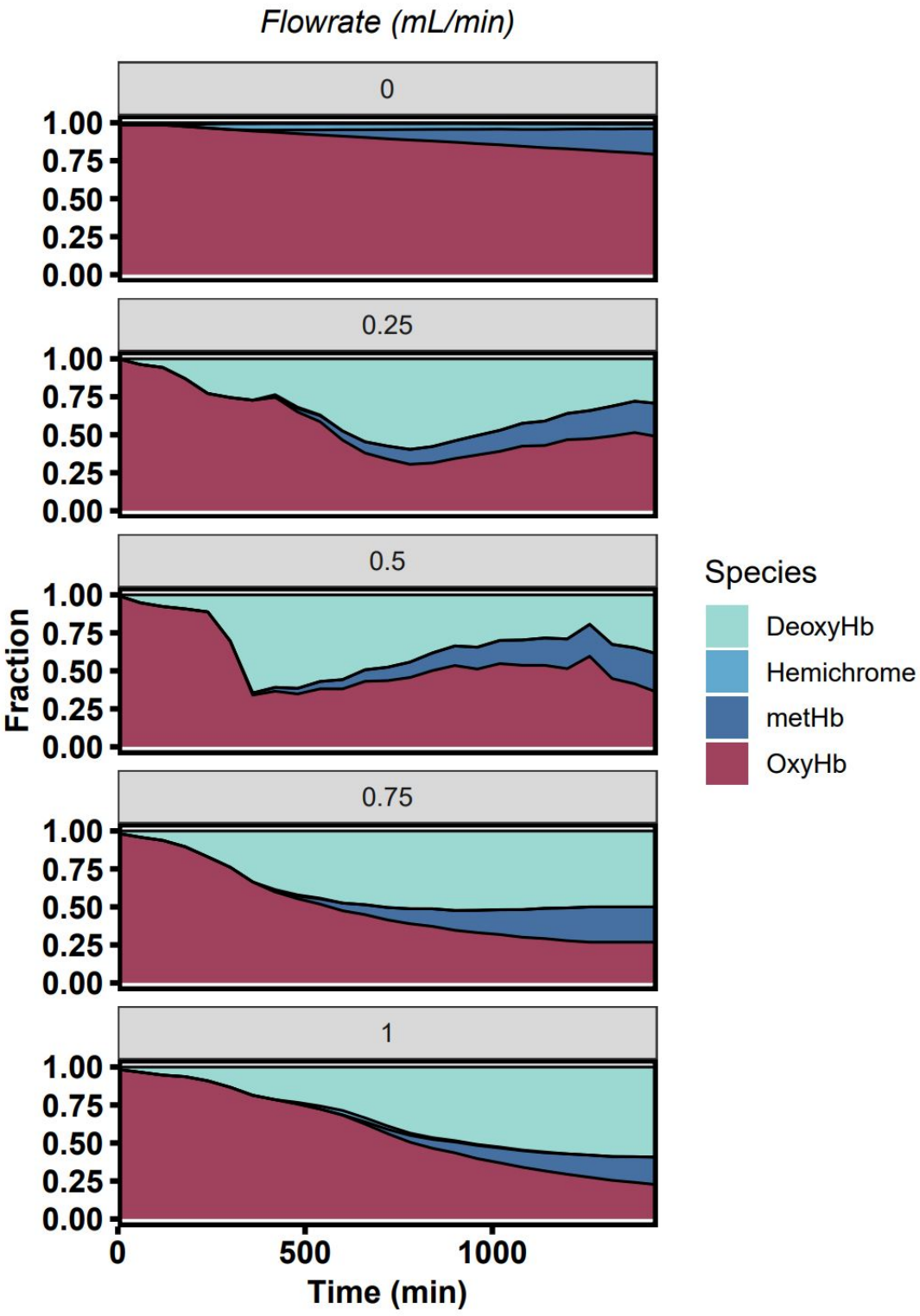

Figure 7: Fractional compositions of Hb species for autoxidation experiments with a gas contactor membrane over a 24 hour period.

The fractional composition graph for the 0 mL/min flowrate is very similar to the dynamic autoxidation fractional composition graph since no N_2 is passing through the circuit. For the 0.25 and 0.50 mL/min flowrates, there was an initial increase and then decrease in deoxyHb fractional composition. With the same gas flowrates, the oxyHb fractional composition decreased and then increased in correspondence. This was probably due to the fact that there was initial deoxygenation occurring but over time a dynamic equilibrium was reached so the oxyHb fractional composition increased. There was a sharper decrease in oxyHb and increase in deoxyHb fractional composition for the 0.25 and 0.50 mL/min flowrates. The oxyHb fractional composition decreased and deoxyHb fractional composition increased consistently throughout the 24 hour time frame. Towards the end of the 24 hour period, the fractional compositions of the Hb species started to level off. This was probably due to the fact that an equilibrium was reached between the deoxygenation via the N_2 in the gas contactor membrane and the ambient air. Table shows the final percent compositions of metHb, oxyHb, and deoxyHb for each of the N_2 gas flowrates.

Table 1: Percent compositions of metHb, oxyHb, and deoxyHb for different N_2 gas flowrates.

N_2 Flowrates (mL/min)	Final metHb %	Final oxyHb %	Final deoxyHb %
0	16.98	79.06	0.94
0.25	21.78	48.84	29.39
0.50	25.26	35.92	38.82
0.75	23.20	26.78	50.02
1.00	18.20	22.51	59.29

The increase in N_2 gas flowrates has an increase in the final deoxyHb percent composition and decrease in the final oxyHb percent composition. However, the metHb fractional composition varied slightly for each gas flowrate but was mostly similar for each of the N_2 flowrates.

The pO_2 levels were monitored using the Neofox dissolved oxygen probe for each of the N_2 gas flowrates. For each of the flowrates, the pO2 levels were graphed over the 24 hour time frame as shown in Figure 8.

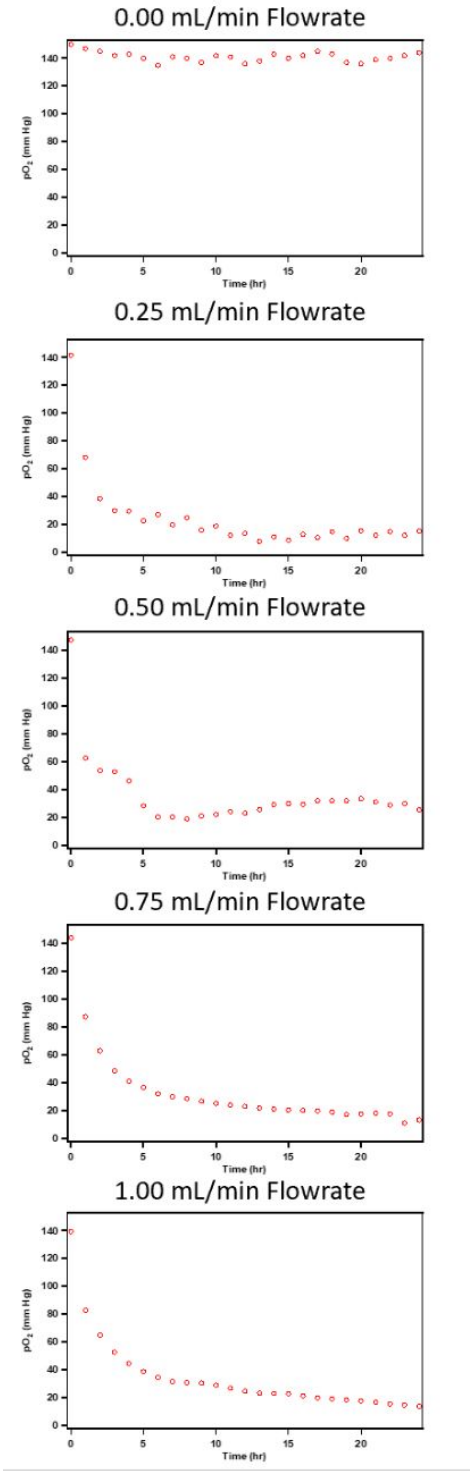

Figure 8: pO_2 levels throughout course of experiment with gas contactor membrane.

The pO2 levels for 0 mL/min were consistent throughout the 24 hour time frame similar to the dynamic autoxidation experiment. The rest of the N_2 flowrates had the pO_2 levels decrease to around 20 mm Hg. For the 0.50 mL/min flowrate, the pO_2 level did not consistently decrease over the 24 hour period.

4 Conclusions

Methemoglobin is a form of hemoglobin that is oxidized and in the ferric state so it is unable to bind to oxygen. Therefore, only minute amounts are allowed to be existent in the body before problems occur. This project was designed to determine how autoxidation in a static and dynamic manner would affect metHb formation. Additionally, the effect of different flowrates of N_2 gas on the fractional compositions of Hb species (oxyHb, deoxyHb, metHb, and hemichrome) was also analyzed. A program called Alchromy was used to analyze these fractional compositions and further analysis was done to obtain the reaction rates for the change in certain Hb species over the 24 hour period.

The static and dynamic autoxidation experiments had very similar fractional compositions for the Hb species over the 24 hour period. They also had very similar reaction rate constants that indicated a first order reaction when graphing the $\ln([Hb^{2+}]/[Hb^{2+}]_0)$ over time. In the t-test conducted, it was found that there was no significant difference between the kinetic rate constants for the static and dynamic autoxidation experiments.

The set of experiments done for the different N_2 gas flowrates showed that there was a significant increase in the fractional composition of deoxyHb and decrease in the fractional composition of oxyHb over the 24 hour period. The change in the fractional composition was not consistent for the flowrates of 0.25 and 0.50 mL/min while for the flowrates 0.75 and 1.00 mL/min it was consistent. The low gas flowrates took more time to stabilize at a certain percent composition of the Hb species.The pO_2 levels decreased as the deoxyHb fractional composition increased for the 0.25, 0.50, 0.75, and 1.00 mL/min N_2 flowrates over the 24 hour time frame.

In terms of future work, a similar type of experiment can be done with the different N_2 gas flowrates but turning on and off the N_2 gas to simulate the oxygenation and deoxygenation loop in the body. The pO_2 levels can be monitored using the dissolved oxygen probe to in the bHb solution. The sample can be dexygenated until it reaches 40 mm Hg, the

standard pO_2 level of deoxygenated blood in the body, and then the N_2 gas can be turned off to allow the sample to oxygenate through oxygen permeable tubing. The experiments conducted for this project can also be tested on other hemoglobin-based oxygen carriers (HBOCs) like polymerized bovine hemoglobin (polybHb), polymerized human hemoglobin (polyHhb), and earthworm hemoglobin (LtEc). In order to simulate physiological conditions in a better manner, enzymes like superoxide dismutase, catalase, or glutathione peroxidase and reducing agents like methylene blue can be added to regulate the formation of metHb and simulate pseudo-physiological conditions.

This closed-circuit can be used to test the HBOCs in pseudo-physiological conditions in the research lab before being sent for in-vivo animal studies. It will give a good indication of how stable and suitable the HBOCs are for the environment within the body.

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6 Appendix A: Additional Figure and Tables

Table 2: MetHb assay results for Alchromy comparison

	dilution	1	2	3	AVG	STD
L3	20	0.553558	0.53714	0.522705	0.309	0.015
		1.01	0.98	0.95	0.98	0.03
		16.2	15.7	15.3	15.8	0.5
L1	10	0.410858	0.410858	0.410858	0.4108582	0
L2	10	0.068497	0.068497	0.068497	0.0684967	0
		0.93	0.93	0.93	0.93	0.00
		91.9%	94.7%	97.4%	94.7%	2.7%

```
Kinetic data report
Using scipy.optimize.curve_fit (non-linear least squares regression)
Version 1.2.1    Richard Hickey          Ohio State University

Filename:       TFF-BHb-36_Nitrite_1-5_L1.xlsx
Reference:      C:/Users/Owner/Desktop/Alchromy-master/refs_with_nitrite.dat
Wavelengths:    450-700
File note:

Start and ending composition (percent)
                    initial        final
Hemichrome      4.52075e-09  4.75688e-07
OxyHb           1.32545e-10  7.98457e-09
metHb               96.5554      97.9955
DeoxyHb         7.52874e-06  3.93127e-07
MetNitrite_Adj  1.44293e-09  1.06828e-07
NaNO2           1.44293e-09  1.06828e-07
HbNO                3.44461       2.0045
Component maximum and mimimums:
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Figure 9: Alchromy output to compare to MetHb assay